I-053 Analysis of Carbohydrate Production in Response to Stasis in *Desulfovibrio vulgaris* and Implications for Biofilm Formation

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Abstract:

Desulfovibrio vulgaris is an anaerobic, δ-*Proteobacterium* that can reduce toxic heavy metals such as chromium and uranium. *D. vulgaris* has become an important model system for bioremediation by sulfate-reducing bacteria, and much work has focused on the biochemical processes that mediate sulfate and heavy metal reduction. However, less is known about the cellular responses to heavy metal and/or environmental stresses in the *Desulfovibrio* species. Initial experiments indicated that *D. vulgaris* Hildenborough (DVH) had a spike in the total carbohydrate level as cells entered stationary-phase growth. A similar spike was observed in the *D. vulgaris* strain ATCC29579, but the total carbohydrate was approximately 2-fold lower. Different methods (e.g., salt/formaldehyde wash, Zwittergent wash, and centrifugation) were evaluated for the determination of internal versus external carbohydrate in *D. vulgaris*, and the best results were obtained with the centrifugation method. The DVH strain had more internal carbohydrate than the ATCC strain (approximately 3-fold), and the ATCC strain appeared to have increased levels of carbohydrate in the culture supernatant (approximately 2-fold). In addition, DVH maintained a higher proportion of total carbohydrate that was localized internally. The data suggested that *D. vulgaris* changes the carbohydrate levels in response to growth conditions with lactate and sulfate as electron donor and acceptor, respectively. The *D. vulgaris* genome contains the presumptive ORFs required for the production and utilization of glycogen, and the megaplasmid contains 10 ORFs annotated as glycosyl transferases or polysaccharide biosynthesis. The data suggested that an increase in carbohydrate occurred during transition to stationary phase, and may play a role in a general stress response. Initial results indicated that growth of DVH and ATCC29579 was inhibited at different concentrations of Cr(VI).

Introduction:

The presented work deals with three strains of *D. vulgaris*, ATCC29579, Hildenborough, and △plasmid. All three strains are considered to be isogenic except △plasmid does not have the 0.2Mb megaplasmid. Initial experiments with three strains have indicated an increased production of carbohydrates as the cells enter into stationary phase, but at varying levels. The increased production of glycogen just before *Prevotella ruminicola* enters into stationary has been demonstrated by Lou et al (1997). There is a possibility that *D. vulgaris* could also be increasing its glycogen concentration just before entering stationary phase, but an alternative explanation is the redistribution of carbon to the outside of the cell. Recent work with different *Desulfovibrio* spp. has shown that biofilms are formed by these SRB, and that the properties of sulfate- and metal-reduction are different compared to the growth of cell suspensions (Dunsmore et al., 2002; Beyenal et al., 2004; Beyenal and Lewandowski, 2004). Biofilm formation has not been reported for *Desulfovibrio vulgaris* ATCC29579, the type strain that served as the source of genomic DNA for sequence determination and construction of our oligonucleotide microarrays. However, our recent results with cultures grown with sulfate and lactate indicate that *D. vulgaris* ATCC29579 can form biofilms.

Annotated genes on the megaplasmid associated with carbohydrate utilization

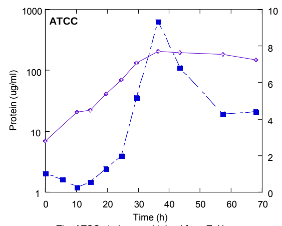
DVUA0040	Polysaccharide biosynthesis protein, punative
DVUA0038	Capsular polysaccharide transport protein
DVUA0037	Sugar transferase domain protein
DVUA0046	glycosyl transferase
DVUA0051	glycosyl transferase
DVUA0054	glycosyl transferase
DVUA0071	glycosyl transferase
DVUA0072	glycosyl transferase
DVUA0081	glycosyl transferase
DVUA0125	Transglycosylase

***D. vulgaris* genes possibly associated with glycogen synthesis and/or carbohydrate transfer as cells enter stationary-phase**

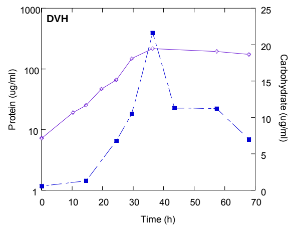
VIMSS ID	Annotated gene	Upregulation
206469	Glucokinase, putative	4.99 ±1.46 z = 2.36±0.87[±]
209685	Sugar transferase domain protein*	4.32 ±0.52 z = 6.07 ±0.84[¶]
208200	transglycosylase SLT domain protein	2.45 ±0.34 z = 2.30 ±0.49[§]
208505	glycosyl transferase, group 2 family protein	2.31 ±0.10 z = 1.65 ±0.58[§]
206369	isoamylase N-terminal domain protein	2.13 ± 0 z = 2.99 [δ]

*Located on plasmid [±]29-61.8h [¶]29 & 43.5h [§]43.5-66.8h [δ]29h

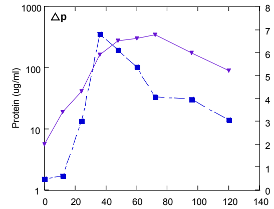
Protein and carbohydrate levels under sulfate-reducing conditions in ATCC, DVH, and △p strains



The ATCC strain was obtained from T. Hazen (LBNL).

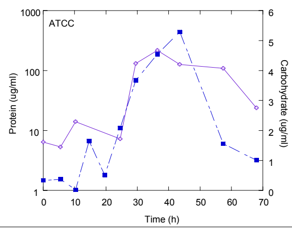


The DVH strain was obtained from the Wall laboratory (originally from H. Peck), and has been cultivated under laboratory conditions for several decades.

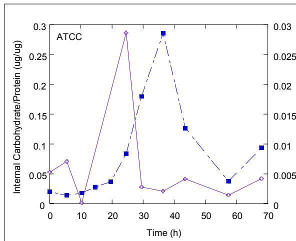


The Δp strain was isolated in the Wall laboratory.

Growth was done in a defined medium (LS4D) that does not contain yeast extract. Protein levels were similar for the three strains, although △p had a slower growth rate. All three strains exhibited an increase in carbohydrate levels that occurred approximately as the cells entered stationary phase. However, the total carbohydrate levels (in cell pellet after centrifugation) were different between the strains. Results are representative of at least three cultures.

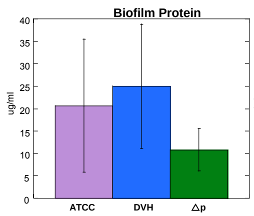


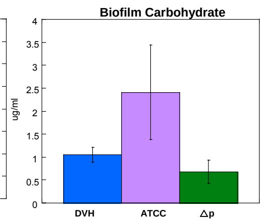
Internal carbohydrate levels after extraction via centrifugation technique. For ATCC, approximately half of the carbohydrate remained at the peak level. A greater portion of the total carbohydrate levels appeared to be internal in DVH (75%), and results indicated that the majority of carbohydrate in △p was internal (90%) (data not shown).

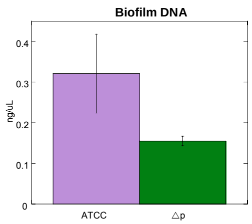


The centrifugation technique was used to extract external carbohydrate, and the remaining carbohydrate levels were determined and compared to the protein levels. The displayed results for ATCC indicated that a transient spike in internal carbohydrate was observed, and a corresponding spike in carbohydrate levels in the culture supernatant were also observed. Data for both DVH and △p suggested that more carbohydrate was located internally, and less carbohydrate was observed in the culture supernatant (data not shown).

Crystal violet staining of biofilm development on glass tubes after *D. vulgaris* cultures reached stationary phase. From left to right, Δp, ATCC, and DVH. CV stain was quantified via a spectrophotometer, and Δp had approximately 3-fold lower biofilm formation.



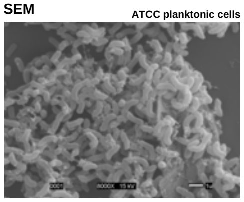




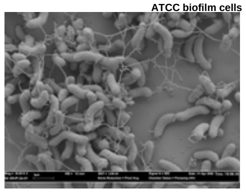
Protein, carbohydrate, and DNA content of biofilms on a glass surface. ATCC biofilms had 2- to 3-fold more protein, carbohydrate, and DNA compared to △p biofilms. These results suggested that the megaplasmid might be involved with carbohydrate allocation and biofilm formation.

SEM

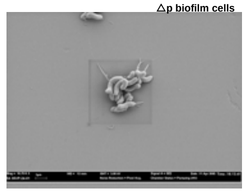
ATCC planktonic cells



ATCC biofilm cells

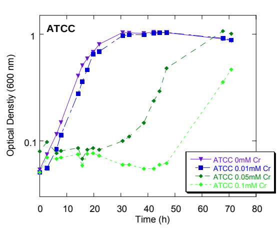


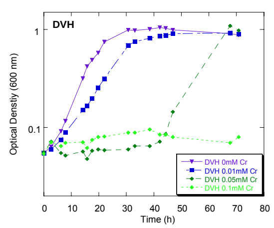
△p biofilm cells

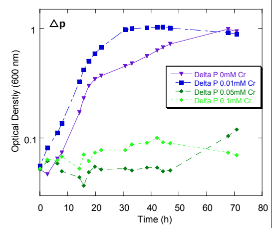


SEM of *D. vulgaris* cells adhered to a glass surface. SEM corroborated the observation that △p cells were deficient in biofilm formation. ATCC biofilm cells appeared to have an altered cell surface compared to planktonic cells and △p biofilms. In addition, the ATCC biofilm cells have extracellular filaments not observed in planktonic cells or △p biofilms. Further work is needed to determine the nature and role of the filaments and the possible role(s) that the megaplasmid plays in biofilm formation.

Growth response of *D. vulgaris* cells to potassium chromate [Cr(VI)]. When 0.01 mM Cr was added to the medium at the time of inoculation, the growth of ATCC was not affected whereas DVH and △p growth was slightly decreased. 0.05 mM Cr caused an approximately 20 h, 30 h, and 40 h lag in ATCC, DVH, and △p, respectively. At a Cr(VI) level of 0.1 mM, ATCC lagged for approximately 40 h, and neither DVH or △p was able to initiate growth. The results indicated that both DVH and △p were deficient in Cr(VI) resistance compared to ATCC, but further work is needed to determine the possible roles of the megaplasmid and biofilm formation.







Materials & Methods

Growth Conditions

D. vulgaris was grown in the minimal media LS4D. LS4D contains 50mM sodium sulfate, 60mM sodium lactate, 8.0mM magnesium chloride, 20mM ammonium chloride, 2.2mM potassium phosphate, 0.6mM calcium chloride, Thauers vitamins, trace minerals, 30mM pipes, 0.064μM resazurin, and 10mN sodium hydroxide to pH to 7.2. Growth occurred in an open system at 30°C continuously sparged with N_2. The growth conditions for the chromium MIC assays are the same except for the concentration of sulfate and lactate in the media and the addition of chromium.

Carbohydrate Extraction

Zwittergent wash: Five hundred microlitters of culture was combined with 100μL of a 1% Zwittergent in 100mM citric acid solution. The mixture was incubated for 20min at 50°C. After incubation, the cells were pelleted at 14, 000 x g for 2 min. Pellets were analyzed for carbohydrates using the cysteine-sulfuric acid method.

Centrifugation: Cell culture (~30 ml) was centrifuged at 8,000 x g for 8 min and the supernatant removed. The pellet was resuspended in 30 ml dH_20, vortexed vigorously for 1 min, and centrifuged at 14,000 x G for 10 min. The supernatant was removed, the pellet resuspended in 2 ml dH_20, and the sample centrifuged at 8,000 x g for 8 min (Brown, 1980; Zhang, 1999).

Protein, Carbohydrate and DNA analysis

Protein levels were measured using the Lowry assay. Carbohydrate levels were measured using the cysteine-sulfuric acid method. DNA was measured by the Quant-iT DNA assay kit (Molecular Probes) in a 96 well plate reader.

Annotated Genes

All information for annotated ORFs was obtained at the VIMSS website, http://www.microbesonline.org/.

Conclusions:

- NaCl/formaldehyde and zwittergent washes caused significant cell lysis in *D. vulgaris* during carbohydrate extraction
- The centrifugation technique was more efficient at removing carbohydrate, and the technique worked better with cultures that had higher OD values
- Carbohydrate levels increased as *D. vulgaris* cells entered stationary phase (ATCC, DVH, △p)
- Approximately half of the carbohydrate in ATCC was localized internally at the highest peak
- Both DVH and △p had a greater proportion of carbohydrate localized internally
- The spike in carbohydrate levels was transient, and a corresponding increase in culture-supernatant carbohydrate was observed
- *D. vulgaris* ATCC adhered to glass surfaces when grown under sulfate-reducing conditions, and the biofilm contained viable cells
- *D. vulgaris* biofilm contained protein, carbohydrate, and DNA, but the ATCC levels were 3- to 5-fold higher compared to the △p strain
- Genes predicted to be involved in glycogen production or the transfer of carbohydrate across the membrane were up-expressed as the cells entered into stationary phase (data not shown)
- The megaplasmid contains numerous ORFs predicted to play a role in carbohydrate transfers and cell interactions (e.g., a unique PilF gene)
- SEM data demonstrated that the △p strain was deficient in biofilm formation and that extracellular structures existed between the ATCC adhered cells that were not observed in planktonic or △p cells
- ATCC was more tolerant of higher concentrations of chromium compared to DVH or △p

Further work is needed to elicit the exact role that the megaplasmid might play in carbohydrate allocation and biofilm formation in *D. vulgaris*